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(54) Title: IMMUNOLOGIC IDENTIFICATION OF CARBOXY TERMINAL SEQUENCES OF ELASTIN IN HUMAN PLASMA USING MONOSPECIFIC ANTIBODIES

(57) Abstract

The present invention relates in part to the isolation and characterization of a portion of the human elastin gene. Through the present invention, sequences have been determined corresponding to the carboxy terminal region of tropoelastin, the primary translation product and biosynthetic intermediate. The protein is terminated by the unusual sequence, GFPGGACLGKACGRKRK. This peptide was synthesized, linked to keyhole limpet hemocyanin, and monospecific antibodies raised in rabbits. The antibodies reacted with peptides derived from human insoluble elastin and were used in an ELISA to quantitate reactive peptides in human plasma samples. COPD patients had significantly higher levels (432 ng/ml equivalents) than control non-smokers (108 ng/ml equivalents).

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IMMUNOLOGIC IDENTIFICATION OF CARBOXY TERMINAL SEQUENCES OF ELASTIN IN HUMAN PLASMA USING MONOSPECIFIC ANTIBODIES

Field of Invention

This invention relates generally to a method for immunologic identification of carboxy terminal sequences of elastin in human plasma using monospecific antibodies.

Chronic obstructive pulmonary disease (COPD) usually develops over many years, and it is not until lung structure and function have been significantly compromised that the disease can be diagnosed with certainty by radiologic and pulmonary function tests.

The connective tissue protein, elastin, is largely responsible for maintaining the elasticity of major blood vessels and lung tissue. In experimental animal models of emphysema, the major emphasis has been on the destruction of the mature elastic fiber by selective proteases administered by aerosol or intra-tracheal instillation. In these systems there is a strong correlation between the production of emphysema and the cleavage of insoluble amorphous lung elastin. Similarly, destruction of the elastic fiber in humans is a likely prerequisite for the development of the disease.

Objects of the Invention

It is a general object of the instant invention to provide a quantitative test of improved specificity that is capable of identifying the presence of lung damage at an early stage, before symptoms develop. Such a quantitative test of improved specificity is useful in identifying individuals who are at risk of developing emphysema. Such a test may also be useful in monitoring the progression of the disease.

Known Prior Art

An early effort in the direction of early detection and for monitoring disease development was the immunologic identification of peptides derived from lung elastin that may appear in the circulation or urine. See Harel S., Janoff A., Yu S.Y., Hurewitz A., Bergofsky E.H. Measurement of Elastin Degradation *in vivo* by Desmosine Radioimmunoassay, *Am Rev Respir Dis* 1980: *122*: 769-73 which shows the use of a radioimmunoassay

to quantitate the characteristic elastin crosslink, desmosine, in human urine and found significantly higher levels in emphysematous patients compared to normal controls. However, more recent studies failed to detect any difference in urine desmosine content between individuals with normal lung function and those with obstructive lung disease. See Davies S.F., Offord K.P., Brown M.G., Campe H., Niewoehner D., Am Rev Respir Dis 1983; 128: 473-5 which found that urine desmosine is unrelated to cigarette smoking or to spirometric function.

In Kucich U., Christner P., Weinbaum G., Rosenbloom J. (hereinafter referred to as Kucich I) Immunologic Identification of Elastin-Derived Peptides in the Serums of Dogs with Experimental Emphysema, Am Rev Respir Dis 1980; 122, 461-5, the present inventors and others used antibodies against elastin-derived peptides (EDP) to detect elastin peptides in the sera of animals with experimental emphysema. See also Darnule T.V., Osman M., Darnule A.T., Mandl I., Turino G.M. Immunologic Detection of Lung Elastin Peptides in the Serum of Rats with Elastin Induced Emphysema, Am Rev Respir Dis 1980; 121: 331.

In an analysis of human plasma by an enzyme-linked immunosorbent assay (ELISA) three of the present inventors with others demonstrated (Kucich U., Christner P. Lippmann M., Kimbel P., Williams G., Rosenbloom J., Weinbaum G.) Utilization of a Peroxidase-Antiperoxidase Complex in an Enzyme-Linked Immunosorbent Assay of Elastin-Derived Peptides in Human Plasma, Am Rev Respir Dis 1985; 131: 709-13, (hereinafter referred to as Kucich II) that significantly higher values of elastin peptides were found in emphysema patients than in individuals with normal lung function. See also article in Am Rev Respir Dis 1983; 127, S28 to S30.

Attention is now called to Darnule T.V., McKee M., Darnule A.T., Turino G.M., Mandl I., Anal Biochem 1982; 122: 302-7, which involves an analysis of human sera by radioimmunoassay which also demonstrated that significantly higher values of elastin peptides were found in emphysema patients than in individuals with normal functions.

While the Kucich II and Darnule studies suggest the possible usefulness of such tests, their value in monitoring the onset and progress of destructive lung disease remains to be proven. In the case of Kucich II, the patients with COPD had elevated peptide levels (127±47ng/ml) as compared to normal non-smokers (58±17 ng/ml), while normal smokers had intermediate values (76±42ng/ml). With a small sample number, PiZZ individuals also appeared to have elevated levels (93±18ng/ml). The aforesaid peptide levels lack specificity, particularly with the large variation of values in patients with COPD.

Summary of the Invention

The present invention involves a comparison with improved specificity to the results obtained with a monospecific antibody generated against a specific amino acid sequence located at the carboxy terminus of human elastin. The levels observed with the present invention clearly set apart the patients with COPD from all others.

Materials and Methods

A. Materials

Goat anti-rabbit (GAR) serum was obtained from Cappel Laboratories (Cochranville, PA) and rabbit peroxidase-antiperoxidase (PAP) complex from Sternberger Meyer Immuno-cytochemicals, Inc. (Jarrettsville, MD). Microtiter plates (Immulon, #2) were obtained from Scientific Accessories (Andalusia, PA). Other chemicals were of reagent grade.

B. Preparation of antigen and antibody

Elastin peptides were prepared as previously described in Kucich I, from the amorphous component of human lung elastin by digestion with purified human neutrophil elastase at a 1:500 ratio of enzyme to elastin (w/w) for 24 hr at 37°C. The peptide, GFPGGACLGKACGRKRK, which composes the carboxy terminus of human elastin was synthesized. The following table sets forth the full amino acids represented by the letter code in the preceding sentence:

AMINO ACID	ONE-LETTER SYMBOL
Glycine	G
Phenylalanine	F
Proline	P
Alanine	A
Cysteine	C
Leucine	L
Lysine	K
Arginine	R
Serine	S

This synthetic peptide GFPGGACLGKACGRKRK was coupled to keyhole limpet hemocyanin (KLH) by using glutaraldehyde described in Baron, M.H., and Baltimore, D., "Antibodies Against a Chemically Synthesized Genome-Linked Protein of Polio Virus React With Native Virus-Specified Proteins, Cell, 1982". The elastin-derived and synthetic peptides were used to generate antibodies in New Zealand white rabbits. See Rosenbloom, J., Kucich, V., Weinbaum, G., Kimbel, P., and Feierstein, M., "Immunologic Identification of Carboxy Terminal Sequences of Elastin in Human Plasma Using Monospecific Antibodies" in "Pulmonary Emphysema and Proteolysis, Volume II" edited by Taylor, J.C., and Mittman C., Academic Press (1986). The IgG fractions were purified by $(NH_4)_2\ SO_4$ precipitation followed by DEAE chromatography.

C. Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter plates were coated with elastin peptides (250 ng/ml), or synthetic peptide (1000 ng/ml), by incubation at 16°C for 24 hr in 0.1 M carbonate, pH 9.6, containing 0.02% NaN_3. Standard curves for the indirect ELISA were generated by incubating 5 ug/ml (micrograms per millimeter) primary antibody (for the elastin-derived peptides) or 7 ug/ml primary antibody (for the synthetic peptide) with variable concentrations of competing antigen for 16 hr at 16°C. These reaction mixtures were transferred to the coated wells and incubated at 16°C for 1 hr. The wells were washed with phosphate buffered saline (PBS-Tween 20) and goat antirabbit serum (Cappel Laboratories,

Cochranville, PA) at 1:2000 was added and incubated for 1 hr at room temperature. After washing, the peroxidase-antiperoxidase complex (Sternberger Meyer Immunocytochemicals, Inc., Jarrettsville, MD) at a dilution of 1:2000 in PBS-Tween 20 was added to the wells for 30 min at room temperature. The wells were washed with PBS-Tween 20 and then a 2 mg/ml solution of o-phenylenediamine and 0.006% H_2O_2 in 0.1 M citrate, pH 4,5, was added. After 45-60 min, the absorbance values were determined at 450 nm using an automatic plate reader (Flow Laboratories, McLean, VA). The unknown plasma samples were analyzed in triplicate at two different concentrations (usually at 1:6 and 1:10 dilutions). See also the procedure and materials of the ELISA of Kucich II.

D. Patient Population Selection

A total of 180 subjects were studied after obtaining informed consent and were divided into three groups: Group I: normal non-smokers (n = 83), Group II: normal smokers (n = 51), and Group III: COPD patients (n = 46), who were either ex-smokers (n = 34) or continued to smoke. Spirometry and lung volumes were determined in each subject by using the M-800 Autobox system produced by SRL, Inc. The results were interpreted using the Intermountain Thoracic Society criteria. See Kanner R.E., Morris A.H., eds. Clinical Pulmonary Function Testing, Intermountain Thoracic Society, Salt Lake City 1975; Intermountain Thoracic Society, Publishers. In addition, each patient filled out a detailed respiratory history questionnaire and a complete physical examination was performed. All smokers had smoked at least 1 pack of cigarettes/day for a period exceeding one year. Subjects with other pulmonary diseases such as asthma or active infection were excluded. Those subjects, both smoker and non-smokers, identified as normal were characterized as such on the basis of spirometry, history, physical and radiologic examinations.

III. Brief Description of the Drawings

Fig. 1 is comprised of three separate plots of plasma elastin peptide levels in control non-smokers, smokers and

emphysema patients as done by a prior method (Kucich II). Plasma elastin peptide levels were measured in triplicate at two different plasma dilutions. Each point represents the average peptide level for all determinations performed on each sample.

Fig. 2 is a standard curve for an indirect ELISA. The assay was carried out as described in Material and Methods as set forth hereinabove.

IV. Results

A. Plasma Elastin-Derived Peptide Levels in Controls, Smokers and Emphysema Subjects

It has been previously proven as set forth in Kucich II, that the elastin-derived peptide (EDP) levels in individual subjects remained relatively constant over a time interval of several months, suggesting that a single blood sample was representative with respect to EDP levels for that individual within that time period. Furthermore, there did not appear to be any age or sex dependence of the EDP levels in individuals with normal lung function whether smokers or non-smokers. See Kucich II.

Figure I of the drawing illustrates the results of EDP measurements in normal non-smokers, smokers and COPD patients. These results clearly demonstrate that on an average, individuals with emphysema have significantly higher levels of elastin-derived peptides compared to normal non-smokers. In addition, the average peptide level of normal smokers is intermediate between the other two groups. While the great majority of normal smokers had elastin levels similar to the non-smoker, there is a small (20%) but significant group of normal smokers who had peptide levels, that is values far greater than 90 ng/ml (90 nanograms per milliliter), in the range of the emphysema group. These data suggest that this asymptomatic group of smokers may have lung elastin breakdown in excess of normal and be at risk of developing COPD.

B. Use of Monospecific Antibody Generated Against a Unique Amino Acid Sequence

The antibodies used in the experiments described above were directed against a complex mixture of peptides, and thus the

observed reactivity in the plasma is the sum of the reactivity of an unspecified number of antigenic determinants. In order to confirm the results and to begin to identify individual circulating determinants, there has been generated antibodies against a defined amino acid sequence which forms the carboxy terminus of human elastin. This sequence was determined from sequencing of a portion of the human elastin gene. An indirect ELISA was established and a standard curve is illustrated in Figure 2. The assay is useful in the 30-2500 ng/ml range. There have also been comparisons of the immunoreactive equivalents observed with the monospecific and heterospecific antibodies in a limited number of samples from normal non-smokers of Pi MM alpha-1-proteinase inhibitor phenotype, from COPD individuals with Pi MM phenotype, and from individuals with Pi ZZ phenotype. The results found in Table I hereinafter, demonstrate a significant elevation in antigenic reactivity using the monospecific antibody in samples from subjects with COPD (average of 432 ng/ml equivalents) compared to controls (108 ng/ml equivalents). Of course, the absolute values, measured in a particular sample as antigenically reactive equivalents, varied between the two assays. Interestingly and possibly of considerable importance, six patients with ZZ phenotype showed significant elevation of EDP.

Introduction to Table I

The assay of EDP by a prior method (Kucich II) has shown a significantly elevated level ($p < 0.001$) in COPD patients (127 ± 47 ng/ml) compared to normal non-smokers (58 ± 17 ng/ml). However, the aforesaid peptide levels lack specificity, particularly with the large variation of values in patients with COPD.

Normal smokers in the foregoing test group had a comparatively small elevation (76 ± 42 ng/ml). Of particular interest was the selected subgroup of these smokers with levels above 90 ng/ml). It is possible these smokers may have a high risk of developing COPD. Indeed, preliminary results, Kucich U., Abrams W.R., Christner P., Rosenbloom J., Kimbel P., Weinbaum G.,

"Molecular Weight Distribution of Elastin Peptides in Plasmas from Human Non Dis 1984; 129: A 307, suggest that a greater pre-preponderance of lower molecular weight peptides may be found in the plasma of smokers with elevated EDP levels compared to the distribution of peptides found in normal non-smokers or smokers with low EDP. This raises the possibility that these smokers with elevated EDP may be degrading elastin in an abnormal fashion.

TABLE I: IMMUNOREACTIVE PEPTIDES IN HUMAN PLASMA

Sample	Elastin peptides (ng/ml)* Monospecific Antibody	Polyclonal Antibody
Control	135	64
Control	115	45
Control	100	64
Control	106	47
Control	86	64
Ave.	108	57
COPD	278	110
COPD	560	129
COPD	542	101
COPD	362	101
COPD	415	101
Ave.	432	107
ZZ		104
ZZ		84
ZZ		70
ZZ		109
ZZ		108
	Ave.	95

*Immunologic reactive equivalents were measured by ELISA as described in Materials and Methods.

In the analysis of EDP, both the antibodies and the antigenic determinants being measured are heterogeneous. This complexity raises difficulties in comparing values in different individuals and, in addition, raises the possibility of

non-specific cross reactivity with an unrelated circulating antigen. In an attempt to minimize these problems, a program of measurement has been initiated using monospecific antibodies generated against defined amino acid sequences in human elastin. Preliminary experiments with one of these antibodies were encouraging in that immunoreactivity was elevated in COPD patients. The fact that these values were apparently higher than those obtained using the antibodies to EDP is not disturbing, since only immunoreactive equivalents in the plasma samples are being measured.

Thus, through the method of the present invention as shown in Table I, it was determined that COPD patients had significantly higher levels (432 ng/ml equivalents) than control non-smokers (108 ng/ml equivalents) and six patients with ZZ phenotype showed significant elevation of EDP.

Without further elaboration, the foregoing will so fully illustrate my invention that others may, by applying current or future knowledge, readily adopt the same for use under various conditions of service.

What is claimed as the invention is:

1. A method for immunologic detection of elastin-derived peptides in human plasma comprising preparing a synthetic peptide by synthesizing the human elastin gene terminated by the unusual sequence GFPGGACLGKACGRKRK, preparing elastin peptides from the amorphous component of human lung elastin, using said synthetic peptide and said elastin-derived peptides to generate separately maintained antibodies, said synthetic peptide and said elastin-derived peptides being used in an indirect ELISA to quantitate the elastin-derived peptides in a human plasma sample, and comparing the results obtained with an established standard.

2. The method of Claim 1 wherein said test person is suspected of being affiliated with a chronic obstructive pulmonary disease (COPD).

3. A method for immunologic detection of elastin-derived peptides in human plasma comprising preparing a synthetic peptide by synthesizing the human elastin gene terminated by the unusual sequence GFPGGACLGKACGRKRK, preparing elastin peptides from the amorphous component of human lung elastin, linking the synthetic peptide to keyhole limpet hemocyanin by using said synthetic peptide to generate antibodies in rabbits, linking the elastin-derived peptides to keyhole limpet hemocyanin by using said elastin-derived peptides to generate antibodies in rabbits, the antibodies from said synthetic peptide and said elastin-derived peptides being used in an indirect ELISA to quantitate the elastin-derived peptides in a human plasma sample, and comparing the results obtained with an established standard.

4. The method of Claim 3 wherein said test person is suspected of being affiliated with a chronic obstructive pulmonary disease.

5. A kit for carrying out a method for immunologic detection of elastin-derived peptides in human plasma, said kit comprising a synthetic peptide prepared by synthesizing the human elastin gene terminated by the unusual sequence GFPGGACLGKACGRKRK, said kit being usable with elastin peptides prepared from the amorphous component of human lung elastin, said kit including means to use said synthetic peptide and said elastin-derived peptides to generate separately maintained antibodies, said kit further including means to use said synthetic peptide and said elastin-derived peptides in an indirect ELISA to quantitate the elastin-derived peptides in a human plasma sample, and means to compare the results obtained with an established standard.

FIG. 1

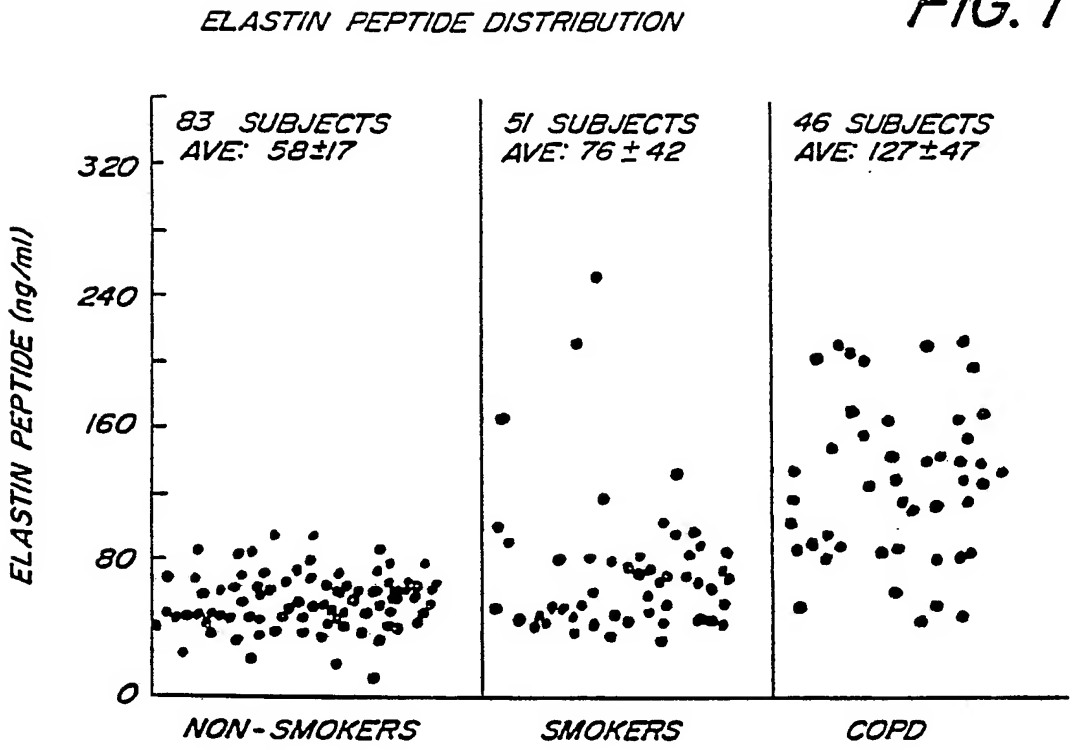
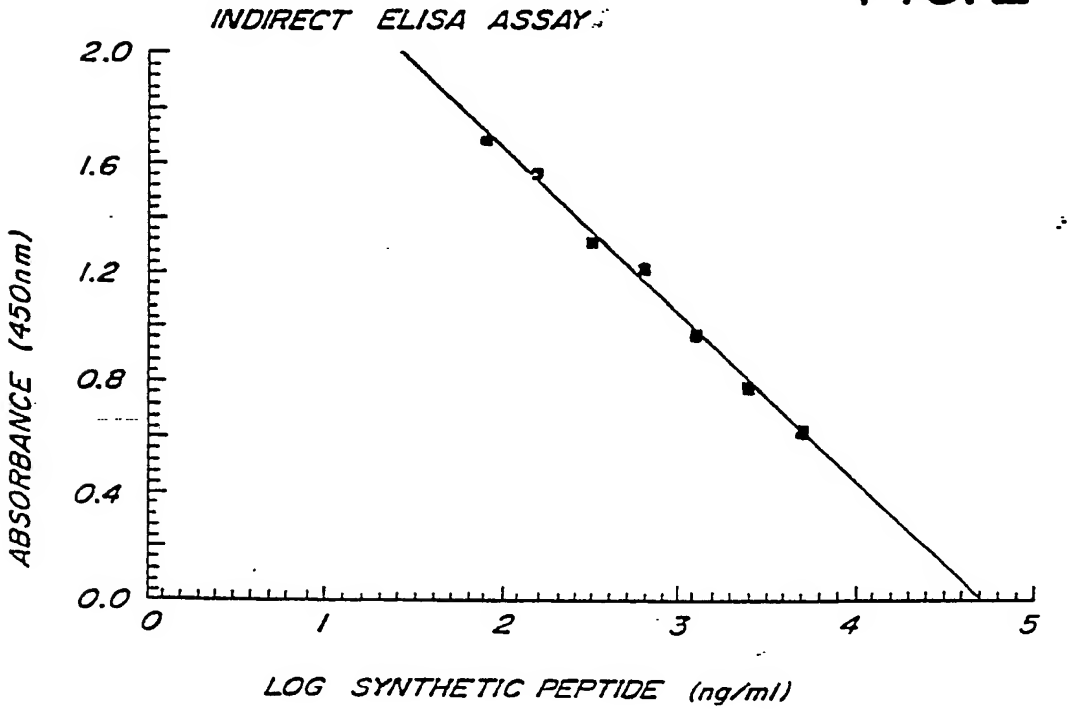

ELASTIN PEPTIDE DISTRIBUTION
83 SUBJECTS
AVE: 58±17
51 SUBJECTS
AVE: 76 ± 42
46 SUBJECTS
AVE: 127±47
ELASTIN PEPTIDE (ng/ml)
320
240
160
80
0
NON-SMOKERS
SMOKERS
COPD

FIG. 2


INDIRECT ELISA ASSAY
ABSORBANCE (450nm)
2.0
1.6
1.2
0.8
0.4
0.0
0
1
2
3
4
5
LOG SYNTHETIC PEPTIDE (ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02685

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): G01N 33/535
U.S. CL.: 435/7

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/7,810 436/518,811,822 530/326, 387, 806

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Computer Search: Chemical Abstracts 1967-1988, Intelli Genetics

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,P Y	Chemical Abstracts, Vol. 107, No. 21, issued 23 November 1987 (Columbus, Ohio, USA), J. Rosenbloom, "Immunologic Identification of Carboxy Terminal Sequences of Elastin In Human Plasma Using Monospecific Antibodies", see page 417, column 1, abstract No. 194478h, Pulm. Emphysema Proteolysis, 1986, [Conf.]. 1986 (Pub. 1987), 245-54.	1,2,5 3-4
Y	American Review of Respiratory Disease, Vol. 131, issued 1985 (New York, New York, USA), U. Kucich, "Utilization of a Peroxidase Antiperoxidase Complex In An Enzyme-Linked Immunosorbent Assay of Elastin-Derived Peptides In Human Plasma", see page 709, column 3, lines 5-14 and page 710, column 1, lines 17-56.	1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 October 1988	08 DEC 1988
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Esther M. Kepplinger ESTHER M. KEPPLINGER

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Archives of Biochemistry And Biophysics, Vol. 241, No. 2, issued September 1985 (New York, New York, USA), K. Yoon, "Analysis of The 3' Region of The Sheep Elastin Gene", see page 688, column 1, line 12 - column 2, line 3, Figure 4 and page 690, lines 1-17.	1-5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.